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ARTICLE

Allelic divergence and cultivar-specific SSR alleles revealed by capillary electrophoresis using fluorescence-labeled SSR markers in sugarcane

Amaresh Chandra, Michael P. Grisham, and Yong-Bao Pan

Abstract: Though sugarcane cultivars (*Saccharum* spp. hybrids) are complex aneupolyploid hybrids, genetic evaluation and tracking of clone- or cultivar-specific alleles become possible through capillary electrophoresis (CE) using fluorescence-labeled SSR markers. Twenty-four sugarcane cultivars, 12 each from India and the USA, were genetically assessed using 21 fluorescence-labeled polymorphic SSR markers. These markers primed the amplification of 213 alleles. Of these alleles, 161 were common to both Indian and US cultivars, 25 were specific to the Indian cultivars, and 27 were observed only in the US cultivars. Only 10 alleles were monomorphic. A high level of heterozygosity was observed in both Indian (82.4%) and US (91.1%) cultivars resulting in average polymorphism information content (PIC) values of 0.66 and 0.77 and marker index (MI) values of 5.07 and 5.58, respectively. Pearson correlation between PIC and MI was significant in both sets of cultivars (r = 0.58 and 0.69). UPGMA clustering separated cultivars into three distinct clusters at 59% homology level. These results propose the potential utility of six Indian cultivar-specific SSR alleles (mSSCIR3_182, SMC486CG_229, SMC36BUQ_125, mSSCIR74_216, SMC334BS_154, and mSSCIR43_238) in sugarcane breeding, vis a vis transporting CE-based evaluation in clone or variety identity testing, cross fidelity assessments, and genetic relatedness among species of the genus *Saccharum* and related genera.

Key words: capillary electrophoresis, cultivar-specific alleles, genetic diversity, microsatellites, sugarcane.

Résumé : Bien que les cultivars de canne à sucre (hybrides de *Saccharum* spp.) soient des hybrides complexes aneu-polyploïdes, l'évaluation et le suivi d'allèles spécifiques d'un cultivar ou d'un clone sont devenus possibles via l'électrophorèse capillaire (CE) et le marquage à la fluorescence des marqueurs SSR. Vingt-quatre cultivars de canne à sucre, 12 chacun de l'Inde et des États-Unis, ont été analysés génétiquement à l'aide de 21 marqueurs SSR polymorphes marqués à la fluorescence. Ces marqueurs ont permis d'amplifier 213 allèles. De ceux-ci 161 étaient communs aux cultivars indiens et américains, 25 étaient spécifiques des cultivars indiens et 27 ont été observés uniquement au sein des cultivars américains. Seuls 10 allèles étaient monomorphes. Un fort degré d'hétérozygotie a été observé tant au sein des cultivars indiens (82,4 %) qu'américains (91,1 %) ce qui a mené respectivement à des indices polymorphisme (PIC) moyens de 0,66 et 0,77 et à des indices de marqueur (MI) de 5,07 et 5,58. Les corrélations de Pearson entre les indices PIC et MI étaient significatives dans les deux cas (r = 0,58 et 0,69). Des analyses de groupement UPGMA ont séparé les cultivars en trois groupes distincts à un niveau d'homologie de 59 %. Ces résultats suggèrent que six allèles SSR spécifiques des cultivars indiens (mSSCIR3_182, SMC486CG_229, SMC36BUQ_125, mSSCIR74_216, SMC334BS_154 et mSSCIR43_238) seraient potentiellement utiles en sélection chez la canne à sucre pour l'identification des clones ou variétés, pour la validation des croisements et pour l'étude des relations de parenté génétique parmi les espèces du genre *Saccharum* et les genres apparentés. [Traduit par la Rédaction]

Mots-clés : électrophorèse capillaire, allèles spécifiques de cultivars, diversité génétique, microsatellites, canne à sucre.

Introduction

Sugarcane (*Saccharum* spp. hybrids) is an important commercial crop grown in over 100 countries. Approximately 70% of the world's sugar (Lakshmanan et al. 2005) and >35% of alcohol (Parida et al. 2010; OECD-FAO 2011) production come from sugarcane with India being the second largest cane producer in the world. India grows sugarcane in a wide range of agro-climatic zones over five million hectares and holds the richest collection of sugarcane germplasm at Coimbatore, India. Since Coimbatore has this diverse collection at a suitable environment for natural flowering, and breeders from all over India perform sugarcane crossing and breeding there, genetic evaluation of this diverse germplasm would be beneficial. Modern sugarcane cultivars are mainly derived from interspecific crosses between the noble cane *Saccharum officinarum* (2n = 80) and the wild species *Saccharum spontaneum* (2n = 40–128). Worldwide sugarcane breeding is considered a tedious and cumbersome process, which usually takes 12–14 years to release a variety or cultivar. During this lengthy breeding process, sugarcane breeders usually maintain the identities of their clones with morphological traits but some of these traits are influenced by environments and distinct clones may look similar when grown in similar environments (Pan et al. 2007). This could compound and lead to potential mixing or mislabeling of clones that are either grown in vicinity of field evaluation plots or on crossing carts. Additionally, vegetative seed source, in the form of either whole stalks or pieces of three-node

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A. Chandra. USDA–ARS, MSA, Sugarcane Research Laboratory, Houma, LA 70360, USA; Indian Institute of Sugarcane Research, Lucknow-226002, India.
M.P. Grisham and Y.-B. Pan. USDA–ARS, MSA, Sugarcane Research Laboratory, Houma, LA 70360, USA.
Corresponding author: Yong-Bao Pan (e-mail: YongBao.Pan@ars.usda.gov).

setts, leads to the cutting and transport of huge amounts of cane that sometimes may also cause mixing of varieties.

Sugarcane research is often impeded by its complex aneuploid genome, narrow gene pool, and poor flowering fertility, caused by genetic recombination as well as long breeding selection cycle. Identifying distinct alleles and transferring these into elite clones is preferred, especially when many alleles are involved as in the case of sugarcane. The high level of ploidy further complicates the situation, as individual genotypes can have multiple alleles at one locus, and loci are also likely to be duplicated. In contrast to cereal crops, the progress in sugarcane genomics is considerably slow. Nevertheless, the key turning points in the recent past, apart from the development of simple sequence repeats (SSR or microsatellite) and conserved-intron scanning primer (CISP) markers, have been the better understanding of the evolutionary origin and genome structure, development and accumulation of important resources such as genetic maps, large ESTs (http://sucest-fun.org/en/projects/sucest-fun/sucest-fun-database), and availability of BAC libraries. Among the molecular markers, the SSR marker is still preferred in molecular breeding work because of its repeatability, its co-dominance, and its economic value once developed. However, multi-allelic representation in sugarcane has made it difficult to score DNA fragments precisely on agarose or polyacrylamide gels. Because of these problems, even SSR markers, which are otherwise regarded as co-dominant markers, are treated as dominant markers in sugarcane, as it is seldom clear whether they represent unique alleles at a single locus or duplicated loci (Butterfield et al. 2004). Therefore, there is a need of improved tools to precisely score and define alleles of precise size in sugarcane. Pan (2006) has demonstrated the utility of 5′-end fluorescence-labeled forward SSR primers in conjunction with capillary electrophoresis (CE) in sugarcane germplasm evaluation and variety identity testing. On a CE–SSR genotyping platform, the sizes of PCR amplicons are computed accurately by calibration against 16 fluorescence-labeled DNA size standards. Based on PCR robustness, presence of regular peaks, and polymorphism information content (PIC), a set of 21 SSR primer pairs was selected for distinguishing among US sugarcane commercial breeding clones and cultivars (Pan et al. 2007). Researchers have detected mislabeling of some commercial clones grown at different geographical locations. However, these studies were largely limited to cultivars from the USA and China (Pan et al. 2007; Chen et al. 2009).

Both restriction- and PCR-based molecular markers have been used to study the genetic diversity among species of the genus *Saccharum* and hybrid populations (D'Hont et al. 1993; Al-Janabi et al. 1994; Harvey and Botha 1996; Nair et al. 1999; Pan et al. 2000; Alwala et al. 2006). Since 2000, both EST- and genomic-based SSR markers have been developed and utilized in genetic diversity and population structure studies involving Indian sugarcane cultivars (Parida et al. 2009; Singh et al. 2013). Some SSR markers have been tested with low and high sugar clones, including different species of *Saccharum* (Singh et al. 2005, 2008). Cross-genus SSR markers have been used for fingerprinting and genetic diversity and AFLP markers for genetic diversity and phenetic organization in the *Saccharum* complex that included species of *Erianthus* and tropical and sub-tropical Indian sugarcane cultivars (Selvi et al. 2003, 2005, 2006). CISP markers utilizing comparative genomics tools have been generated and utilized to study the phylogenic relationship in species of *Saccharum* and sugarcane cultivars (Khan et al. 2011; Chandra et al. 2013). Recently, target region amplification polymorphism (TRAP) and single nucleotide polymorphism (SNP) markers have been also used in genetic evaluation of *Saccharum* and related genera (Devarumath et al. 2013). However, most of these studies were based on agarose or polyacrylamide gel electrophoresis detection systems. The present study was undertaken to genetically compare 12 well-adopted Indian sugarcane cultivars with 12 leading Louisiana sugarcane cultivars using the CE–SSR genotyping system and to identify alleles that are unique to the Indian or US cultivars.

Table 1. A list of the sugarcane cultivars, their parentage, and place of origin used for capillary electrophoresis with fluorescence-labeled SSR markers.

No.	Cultivar	Parentage	Place of origin
1	CoLk8001	Co62174 × Co1148	Lucknow, India
2	CoLk8102	Co1158GC	Lucknow, India
3	CoLk94184	CoLk8001 self	Lucknow, India
4	CoLk9606	Co7227GC	Lucknow (clone)
5	CoLk9617	Co62399 × BO91	Lucknow (clone)
6	CoS767	Co419 × Co313	Shahjahanpur, India
7	CoS95255	Co1158 × Co62198	Shahjahanpur, India
8	CoS97264	Co1158 × CoS510	Shahjahanpur, India
9	CoSe92423	BO91 × Co453	Seorahi, India
10	CoJ64	Co976 × Co617	Jalandhar, India
11	Co1148	P4383 × Co301	Karnal, India
12	BO91	BO55 × BO43	Bihar-Orissa, India
13	LCP85-384	CP77-310 × CP77-407	Louisiana, USA
14	Ho05-961	CP83-644 × TucCP77-42	Louisiana, USA
15	Ho95-988	CP86-941 × US89-12	Louisiana, USA
16	HoCP91-555	CP83-644 × LCP82-94	Louisiana, USA
17	HoCP00-950	HoCP93-750 × HoCP92-676	Louisiana, USA
18	HoCP04-838	HoCP85-845 × LCP85-384	Louisiana, USA
19	HoCP85-845	CP72-370 × CP77-403	Louisiana, USA
20	HoCP96-540	LCP86-454 × LCP85-384	Louisiana, USA
21	L97-128	LCP81-10 × LCP85-384	Louisiana, USA
22	L01-283	L93-365 × LCP85-384	Louisiana, USA
23	L99-226	CP89-846 × LCP81-30	Louisiana, USA
24	L99-233	CP79-348 × HoCP91-552	Louisiana, USA

Materials and methods

Plant material and DNA extraction

Twelve Indian sugarcane cultivars, namely CoLk8102, CoLk8001, CoS767, CoLk9606, CoLk9617, CoS95255, BO91, CoJ64, Co1148, CoS97264, CoSe92423, and CoLk94184, and 12 US sugarcane cultivars, namely Ho95-988, Ho05-961, HoCP85-845, HoCP91-555, HoCP96-540, HoCP00-950, HoCP04-838, L97-128, L01-283, L99-226, L99-233, and LCP85-384, were involved in this study. The Indian cultivars were grown at the Indian Institute of Sugarcane Research, Lucknow, India, following normal agronomical practices. Fresh leaves were collected in liquid nitrogen before DNA extraction following the method described by Doyle and Doyle (1990). The US cultivars were grown at the breeding nursery at the Ardoyne Farm of the USDA–ARS, Sugarcane Research Laboratory, Houma, Louisiana, USA. Genomic DNA of US cultivars was extracted from the leaf tissue according to Pan et al. (2000). DNA concentrations were determined on a NanoDrop 1000 spectrophotometer (NanoDrop, Bethesda, Md., USA) followed by equilibration through agarose gel electrophoresis. The final concentration of DNA was adjusted to 10 ng/μL. Details of the cultivars used are given in Table 1.

SSR markers, PCR, and fluorescence-based capillary electrophoresis

Twenty-one SSR markers that gave consistent banding patterns from sugarcane cultivars were selected (Pan 2006). To facilitate the laser detection of amplified fragments during the CE process, the forward primers of seven SSR markers (SMC486CG, SMC569CS, mSSCIR3, mSSCIR43, SMC278CS, SMC334BS, and SMC597CS) were labeled with fluorescence dye 6-FAM, six SSR markers (SMC119CG, SMC31CUQ, mSSCIR74, mSSCIR66, SMC1604SA, and SMC703BS) with fluorescence dye VIC, and eight SSR markers (SMC36BUQ, SMC7CUQ, SMC1751CL, SMC22DUQ, SMC24DUQ, SMC851MS, SMC18SA, and SMC336BS) with fluorescence dye NED. SSR genotyping experiments were conducted following a fluorescence- and CE-based high throughput genotyping protocol of Pan et al. (2007). The PCR reaction was performed in a 5 μL reaction mixture consisting of 0.25 μL of genomic DNA, 0.5 μL of 10X buffer, 0.3 μL

Table 2. Detail description of the 21 SSR markers, number of unique alleles amplified, number of mono- and polymorphic alleles, polymorphism information content (PIC), and marker index (MI) in Indian and US cultivars.

No.	SSR marker	Primer sequence (5′→3′)	Repeats	Total no. of alleles (India/USA)	Monomorphic alleles (India/USA)	Polymorphic alleles	Unique alleles	PIC (India/USA)	MI (India/USA)
1	SMC119CG	F:TTCATCTCTAGCCTACCCCAA R:AGCAGCCATTTACCCAGGA	$(TTG)_{12}$	7/5	1/0	6/5	—	0.70/0.62	4.20/3.10
2	SMC1604SA	F:AGGGAAAAGGTAGCCTTGG R:TTCCAACAGACTTGGGTGG	$(TGC)_7$	6/6	2/1	4/5	—	0.50/0.76	1.99/3.80
3	SMC18SA	F:ATTCGGCTCGACCTCGGGAT R:GTCGAAAGGTAGCGTGGTGTTAC	$(CGA)_{10}$	11/11	2/1	9/10	—	0.57/0.87	5.09/8.70
4	SMC24DUQ	F:CGCAACGACATATACACTTCGG R:CGACATCACGGAGCAATCAGT	$(TG)_{13}$	12/11	2/3	10/8	—	0.64/0.71	6.35/5.68
5	SMC278CS	F:TTCTAGTGCCAATCCATCTCAGA R:CATGCCAACTTCCAAACAGACT	$(TG)_{19}$ $(AG)_{25}$	9/8	1/0	8/8	—	0.65/0.73	5.19/6.04
6	SMC31CUQ	F:CATGCCAACTTCCAATACAGACT R:AGTGCCAATCCATCTCAGAGA	$(TC)_{10}(AC)_{22}$	9/9	1/0	8/9	—	0.65/0.72	5.19/6.48
7	SMC334BS	F:CAATTCTGACCGTGCAAAGAT R:CGATGAGCTTGATTG CGAATG	$(TG)_{36}$	12/8	0/0	12/8	1	0.92/0.85	11.03/6.80
8	SMC336BS	F:ATTCTAGTGCCAATCCATCTCA R:CATGCCAACTTCCAA ACAGAC	$(TG)_{23}(AG)_{19}$	9/8	1/1	8/7	—	0.66/0.76	5.27/5.32
9	SMC36BUQ	F:GGGTTTCATCTCTAGCCTACC R:TCAGTAGCAGAGTCAGACGCTT	$(TTG)_7$	4/4	1/0	3/4	1	0.75/0.81	2.25/3.24
10	SMC486CG	F:GAAATTGCCTCCCAGGATTA R:CCAACTTGAGAATTGAGATTCG	$(CA)_{34}$	9/7	2/0	7/7	1	0.71/0.59	4.98/4.13
11	SMC569CS	F:GCGATGGTTCCTATGCAACTT R:TTCGTG GCTGAG ATTCACACTA	$(TG)_{37}$	5/6	2/0	3/6	—	0.72/0.86	2.18/5.16
12	SMC7CUQ	F:GCCAAAGCAAGGGTCACTAGA R:AGCTCTATCAGTTGAAACCGA	$(CA)_{10}(C)_4$	7/5	3/1	4/4	—	0.60/0.75	2.41/3.00
13	SMC597CS	F:GCACACCACTCGAATAACGGAT R:AGTATATCGTCCCTGGCATTCA	$(AG)_{31}$	15/11	3/0	12/11	—	0.65/0.83	7.76/9.13
14	SMC703BS	F:GCCTTTCTCCAAACCAATTAGT R:GTTGTTTATGGAATGGTGAGGA	$(CA)_{12}$	12/10	3/1	9/9	—	0.50/0.78	4.48/7.02
15	SMC851MS	F:ACTAAAATGGCAAGGGTGGT R:CGTGAGCCCACATATCATGC	$(AG)_{29}$	9/9	0/1	9/8	—	0.60/0.76	5.44/6.08
16	mSSCIR66	F:AGG GATTTAGCAGCATA R:CACAAATAAACCCAATGA	$(GT)_{43}GC(GT)_6$	9/9	1/0	8/9	—	0.80/0.81	6.42/7.29
17	mSSCIR3	F:ATACTCCCACACCAAATGC R:GGACTACTCCACAATGATGC	$(GT)_{28}$	12/10	1/0	11/10	1	0.77/0.88	8.47/8.80
18	SMC1751CL	F:GCCATGCCCATGCTAAAGAT R:ACGTTGGTCCCGGAACCG	$(TGC)_7$	7/7	2/3	5/4	—	0.63/0.55	3.16/2.20
19	SMC22DUQ	F:CCATTCGACGAAAGCGTCCT R:CAAGCGTTGTGCTGCCGAGT	$(CAG)_5C(AGG)_5$	9/8	2/0	7/8	—	0.66/0.62	4.61/4.96
20	mSSCIR43	F:ATTCAACGATTTTCACGAG R:AACCTAGCAATTTACAAGAG	$(GT)_3(AT)_2(GT)_{29}$	13/11	1/1	12/10	1	0.66/0.75	7.91/7.50
21	mSSCIR74	F:GCGCAAGCCACACTGAGA R:ACGCAACGCAAAACAACG	$(CGC)_9$	7/6	3/2	4/4	1	0.51/0.62	2.03/2.48
Mean total (India/USA)				193/169	34/15	159/154	6	0.66/0.77	5.07/5.58

of 25 mmol/L $MgCl_2$, 0.1 μL of 10 mmol/L dNTPs, 0.41 μL each of 3 pm/μL forward and reverse primers, 0.5 μL of 10 mg/mL BSA-V, 0.5 μL of 100 mg/mL PVP-40, 0.025 μL of 5 U/μL *Taq*, and 2.0 μL of PCR water. The PCR amplification reactions were conducted on a DNA Engine Tetra equipped with four 384-well alpha blocks with heated lids (Bio-Rad Laboratories, Hercules, Calif.) under the program of 95 °C for 15 min; 40 cycles of 94 °C for 15 sec, annealing for 15 sec, and 72 °C for 1 min; final extension at 72 °C for 10 min; and holding at 4 °C. Annealing temperatures varied with SSR markers and were described earlier (Pan 2006). The CE sample plate was prepared by first diluting the amplified SSR DNA fragments and then dispensing in each well 1 μL of the diluted products and 9 μL Hi-Dye formamide solution premixed with the Rox 500 size standards following manufacturer's instruction (Applied Biosystems, Inc., Foster City, Calif.).

Visualization of alleles and data analysis

Fragment analyses of fluorescence-labeled PCR products along with GeneScan-500 (GS500) size standards were conducted on an ABI 3730XL genetic analyzer (Applied Biosystems, Foster City, Calif.) following manufacturer's instructions. During the capillary electrophoresis, the separation processes were recorded automatically into individual GeneScan files, which were then analyzed with GeneMapper software (Applied Biosystems, Inc., Foster City, Calif.). Alleles were manually assigned to regular plus-A fluorescence peaks. Irregular peaks such as minus-A, stutter, and dinosaur tails (Pan et al. 2003*a*) were not scored. Presence of alleles as amplified by 21 SSR markers with different cultivars was manually scored to define the allele's specific to Indian and US cultivars.

Presence of an SSR allele was given a score of A while its absence a score of C. The distribution of SSR alleles produced by all the 21 SSR markers of all 24 cultivars was then recoded into an arbitrary binary sequence of A's or C's in a fixed order. The resulting 13 SSR genotypes were then aligned with DNAMAN software (Lynnon Biosoft, Vaudreuil, Québec, Canada) to generate both a homology tree using the UPGMA method and a phylogenetic tree using the neighbor-joining method that also showed bootstrapping

Fig. 1. Pearson correlation coefficient (*r*) estimates, polymorphism information content (PIC), and marker index (MI) generated by SSR polymorphism marker system with (A) Indian and (B) US cultivars.

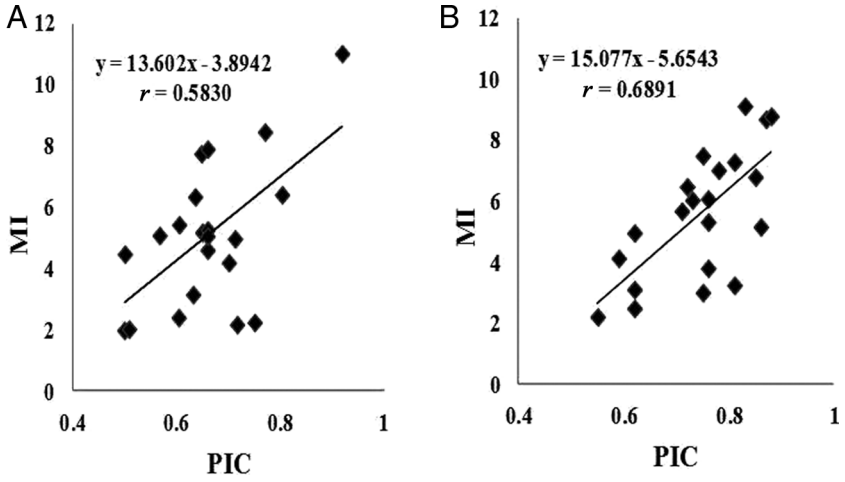


Table 3. Representation of SSR alleles in Indian and US cultivars.

No.	Parameter	Total no. of alleles	Representation (%)
1	Total number of alleles amplified with Indian cultivars only	193	—
2	Total number of alleles amplified with US cultivars only	169	—
3	Total number of alleles amplified	213	—
4	Alleles observed in both Indian and U.S. cultivars	161	75.6
5	Alleles specific to Indian commercial cultivars only (not seen with US cultivars)	25	11.7
6	Alleles specific to US cultivars only (not seen with Indian cultivars)	27	12.7
7	Alleles associated with US commercial cultivars observed in all 12 Indian cultivars	32	18.9
8	US cultivar based allele observed in an Indian cultivar	6	3.6

(confidence) values. The algorithm produced initially a homology matrix based on the sequence variability among molecular identities, and then applied a correction method (Jukes and Cantor 1969) to align progressively all sequences according to the branching order in the phylogenetic tree using the dynamic alignment method. The analytical parameters for the dynamic multiple sequence alignment were set at 10 for gap open penalty, 5 for gap extension penalty, and 40% for delay divergent sequences. Genetic distance was also calculated (Nei 1987). Bootstrap values were obtained upon 1000 trials. PIC was calculated for each marker by applying the formula of Milbourne et al. (1997)

$$\mathrm{PIC} = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of the *j*th allele for marker *i* and summation extends over *n* alleles.

Marker index (MI) was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell et al. 1996).

Results

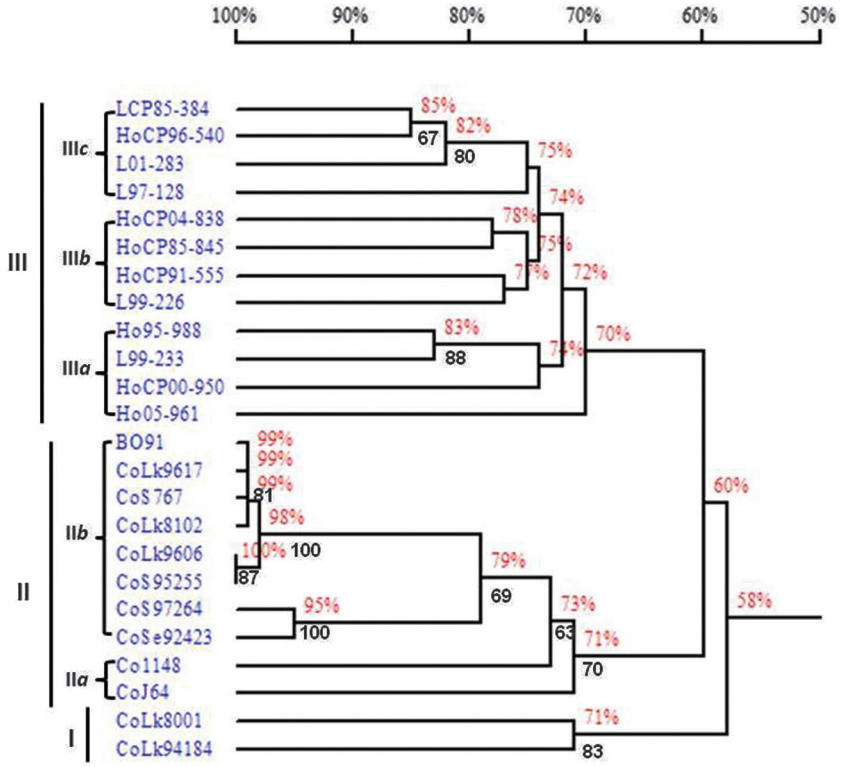
A summary of the alleles produced by the 21 SSR markers across 12 Indian and 12 US cultivars is given in Table 2. The number of alleles produced per SSR marker ranged from 4 (SMC36BUQ) to 15 (SMC597CS) with an average of 9.2. In total, 193 and 169 alleles were scored with Indian and the US cultivars, respectively. The number of monomorphic alleles per SSR marker ranged from 0 to 3, whereas polymorphic alleles ranged from 3 to 12 in the Indian cultivars and from 4 to 11 in the US cultivars. Total polymorphic alleles from Indian and the US cultivars were 159 (82.4%) and 154 (91.1%), respectively. Total monomorphic alleles were 34 (17.6%) and 15 (8.9%), respectively, but only 10 monomorphic alleles were common to all 24 cultivars. Of the total 21 SSR markers, SMC334BS, SMC597CS, and mSSCIR43 generated a maximum 12 polymorphic alleles with Indian cultivars, whereas SMC597CS produced a maximum of 11 such alleles from the US cultivars. The PIC values varied among the SSR markers from 0.50 with SMC1604SA to 0.92 with SMC334BS, with a mean value of 0.66 with Indian cultivars, while the average PIC value with the US cultivars was 0.77. The MI of each SSR marker ranged from 1.99 (SMC1604SA) to 11.03 (SMC334BS) with an average of 5.07 with the Indian cultivars and 5.58 with the US cultivars. The Pearson correlation between the observed PIC and the MI was found to be 0.58 and 0.69 with Indian and the US cultivars, respectively, and considered as significant for the present marker system (Fig. 1). When alleles amplified from both the US and Indian cultivars were taken together, the number of total alleles increased to 213 with this set of SSR markers (Table 3). Of the 213 alleles, 161 were common to both Indian and the US cultivars, 25 were specific to the Indian cultivars, and 27 were observed only in the US cultivars. As many as 32 alleles that were associated with the US cultivars were also observed in all Indian cultivars, whereas only six such alleles were seen in one Indian cultivar.

The greatest genetic distance observed was 0.48 between the US cultivar Ho05-961 and three Indian cultivars (BO91, CoLk9617, and CoLk8102). Among the US cultivars, the highest genetic distance was 0.34 between Ho05-961 and L97-128, while among the Indian cultivars it was 0.45 between BO91 and CoLk8001 (Table 4). When all 24 SSR genotypes were aligned using the DNAMAN software, both homology and phylogenetic trees showed percent homology and genetic distance with bootstrap and confidence values (Fig. 2). Dendrogram separated the US and Indian cultivars into three distinct clusters (I, II, and III) at 59% homology level. Indian cultivars

Table 4. Distance matrix among 24 sugarcane cultivars.

	Cultivars	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	LCP85-384	**0**																							
2	HoCP96-540	0.155	**0**																						
3	L01-283	0.2	0.164	**0**																					
4	L97-128	0.245	0.236	0.273	**0**																				
5	HoCP04-838	0.241	0.232	0.25	0.25	**0**																			
6	HoCP85-845	0.314	0.268	0.241	0.259	0.218	**0**																		
7	HoCP91-555	0.309	0.236	0.273	0.273	0.259	0.223	**0**																	
8	L99-226	0.273	0.218	0.255	0.273	0.241	0.259	0.277	**0**																
9	Ho95-988	0.265	0.256	0.26	0.279	0.311	0.274	0.324	0.292	**0**															
10	L99-233	0.32	0.283	0.256	0.283	0.279	0.279	0.283	0.265	0.174	**0**														
11	HoCP00-950	0.292	0.283	0.301	0.265	0.279	0.279	0.301	0.283	0.247	0.273	**0**													
12	Ho05-961	0.301	0.292	0.269	0.342	0.311	0.265	0.333	0.32	0.255	0.297	0.324	**0**												
13	BO91	0.37	0.388	0.388	0.434	0.42	0.466	0.416	0.425	0.397	0.373	0.391	0.475	**0**											
14	CoLk9617	0.37	0.388	0.388	0.434	0.42	0.457	0.416	0.425	0.397	0.373	0.391	0.475	0.009	**0**										
15	CoS767	0.37	0.379	0.379	0.425	0.411	0.457	0.406	0.416	0.388	0.364	0.382	0.466	0.009	0.009	**0**									
16	CoLk8102	0.37	0.388	0.388	0.425	0.42	0.466	0.416	0.425	0.397	0.373	0.391	0.475	0.009	0.009	0.009	**0**								
17	CoLk9606	0.361	0.37	0.37	0.416	0.402	0.447	0.406	0.416	0.379	0.355	0.373	0.457	0.018	0.018	0.009	0.018	**0**							
18	CoS95255	0.361	0.37	0.37	0.416	0.402	0.447	0.406	0.416	0.379	0.355	0.373	0.457	0.018	0.018	0.009	0.018	0	**0**						
19	CoS97264	0.361	0.37	0.379	0.379	0.384	0.447	0.416	0.397	0.434	0.391	0.4	0.429	0.191	0.2	0.2	0.2	0.2	0.2	**0**					
20	CoSe92423	0.37	0.379	0.406	0.397	0.411	0.447	0.434	0.416	0.443	0.427	0.427	0.447	0.209	0.209	0.218	0.218	0.227	0.227	0.045	**0**				
21	Co1148	0.394	0.436	0.394	0.468	0.422	0.491	0.394	0.431	0.477	0.406	0.443	0.445	0.237	0.237	0.237	0.237	0.247	0.247	0.347	0.365	**0**			
22	CoJ64	0.315	0.324	0.379	0.379	0.347	0.42	0.379	0.361	0.374	0.355	0.336	0.416	0.282	0.282	0.282	0.282	0.291	0.291	0.273	0.291	0.32	**0**		
23	CoLk8001	0.431	0.468	0.408	0.413	0.408	0.408	0.399	0.404	0.427	0.37	0.452	0.376	0.452	0.443	0.443	0.443	0.434	0.434	0.397	0.411	0.39	0.397	**0**	
24	CoLk94184	0.384	0.402	0.42	0.393	0.388	0.452	0.42	0.438	0.429	0.405	0.468	0.425	0.432	0.432	0.432	0.423	0.441	0.441	0.45	0.45	0.32	0.341	0.292	**0**

Fig. 2. Dendrogram (homology tree) based on the alignment of SSR genotypes (213 alleles) of 24 cultivars (12 each from India and USA) with DNAMAN software (Lynmon Biosoft, Vaudreuil, Québec, Canada). The numerical values in percentage showed homology among the cultivars forming different nodes in three major clusters (I, II, and III). Bootstrapping (confidence) values (>60) upon 1000 trials are also given on respective nodes.



were separated in two clusters (I and II), while all the US cultivars were grouped in cluster III. Two Indian cultivars (CoLk8001 and CoLk94184) distinctly formed cluster I and joined with the rest of the cultivars at 58% homology. Clusters II and III joined together at 60% homology. The Indian cultivars of cluster II were further assigned into two subclusters (IIa and IIb), whereas all the US cultivars of cluster III were further assigned into three subclusters (IIIa, IIIb, and IIIc). Cultivars (Co1148 and CoJ64) of subcluster IIa joined to the major Indian subcluster IIb at 79% homology. In subcluster IIb, barring CoS97264 and CoSe92423, the rest of the cultivars showed a very high level of homologies among themselves (>98%). The four cultivars of subcluster IIIb formed two groups. In subcluster IIIc, LCP85-384 and HoCP96-540 cultivars showed an 85% homology. L01-283 joined with these cultivars at 82%, whereas L97-128 joined the subcluster at 75% genetic similarity (Fig. 2). Two Indian cultivars (CoLk9606 and CoS95255) produced identical fingerprints for all 21 SSR markers. These two cultivars joined with four other cultivars (BO91, CoLk9617, CoS767, and CoLk8102) at 98% homology level.

A total of six cultivar-specific SSR alleles were observed with 21 SSR markers. Of these, three alleles, namely SMC486CG_229, SMC36BUQ_125, and mSSCIR74_216, were specific to cultivar CoLk8001, whereas one allele each was observed with CoJ64 (mSSCIR3_182), CoLk94184 (SMC334BS_154), and CoSe92423 (mSSCIR43_238) cultivars (Fig. 3; supplementary data, Fig. S1[1]). Also, six alleles that were predominantly associated with the US cultivars were observed in one of the Indian cultivars. Of these alleles, two (SMC334BS_161 and mSSCIR3_187) were observed in Co1148, three (SMC486CG_227, mSSCIR66_130, and mSSCIR66_134) in CoLk8001, and one (SMC851MS_136) in CoJ64. Two alleles, namely mSSCIR3_202 and mSSCIR3_204, were observed in one US (L99-233) and one Indian (Co1148) cultivar.

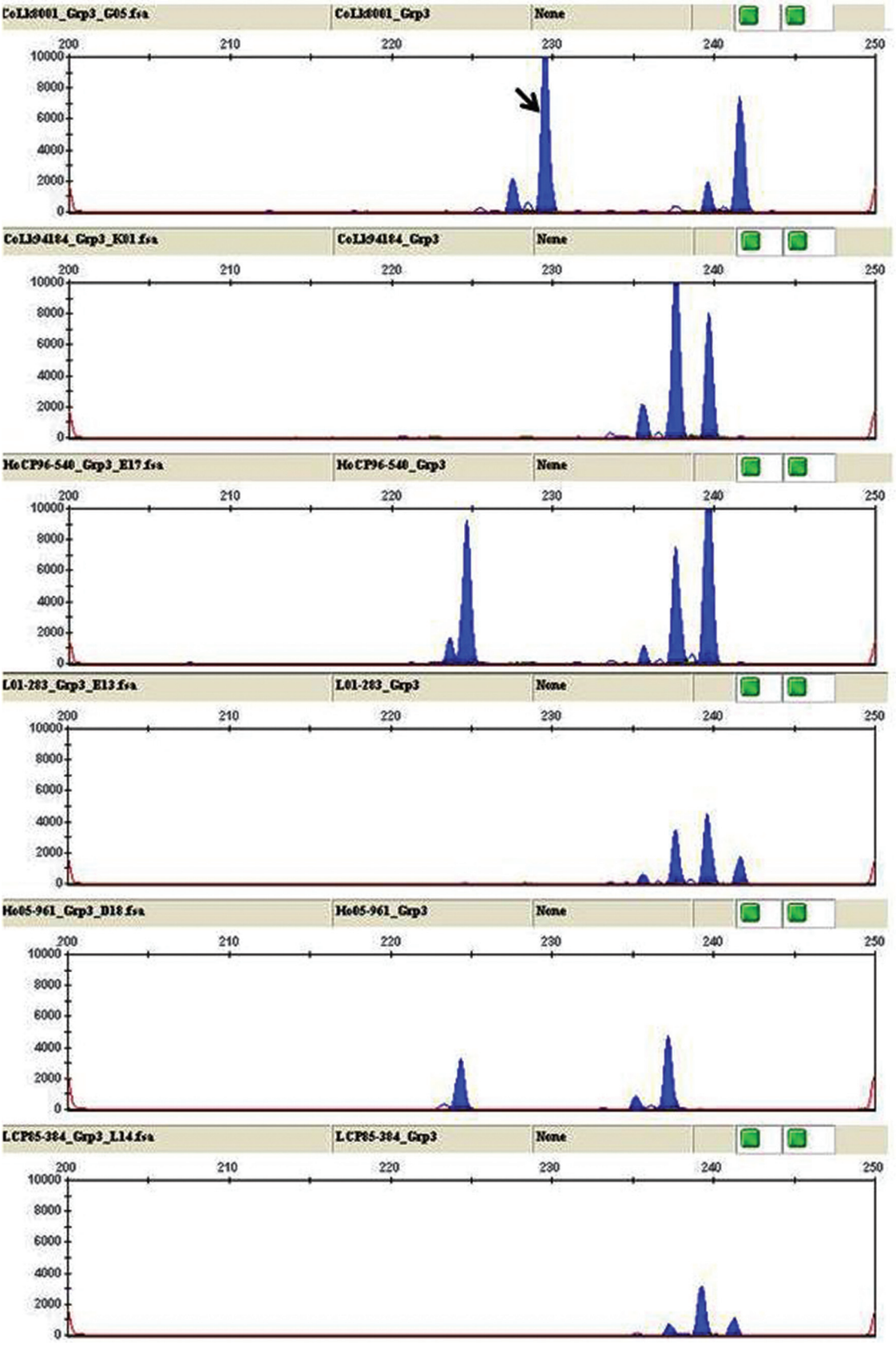
Discussion

In the present study, a high level of allelic divergence is observed among 24 sugarcane cultivars when assessed using a SSR–CE-based detection system. Of the 213 alleles detected, 159 (82.4%) and 154 (91.1%) alleles were polymorphic with Indian and the US cultivars, respectively. Only 10 alleles were monomorphic among all 24 cultivars. The presentation of these alleles resulted in average PIC values of 0.66 and 0.77 and MI values of 5.07 and 5.58 for the Indian and US cultivars, respectively. Also, the higher genetic distance as observed between Indian and the US sugarcane cultivars further entrusted the utility of such a marker system in polymorphism estimation. Among the US cultivars, the highest genetic distance was 0.34 between Ho05-961 and L97-128, while among the Indian cultivars it was 0.45 between BO91 and CoLk8001, indicating more divergence among Indian than those among the US cultivars. The genetic distance of 0.32 was found between the US cultivar LCP85-384 and the Indian cultivar CoJ64. Both cultivars have high sugar content and are widely adopted in their respective countries.

The 21 SSR markers used were highly polymorphic when tested with several US cultivars (Pan 2006). The present study further demonstrated their usefulness with Indian cultivars. Some SSR markers may not always amplify because these SSR markers were developed from specific sugarcane clones; for example, the SMC- and the mSSCIR-series SSR markers were developed from Australian cultivar Q124 and Reunion cultivar R570, respectively. However,

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2014-0072.

Fig. 3. A cultivar-specific allele (SMC486CG_229) obtained with the Indian cultivar CoLk8001. The arrow depicts the specific allele.



once these markers were tested with a reasonable number of commercial and breeding clones, such nonamplification problems can be avoided as in the present study where all 21 SSR markers amplified with a reasonable number of alleles and an average of 9.2 alleles per marker. This is also considered as an advantage when dealing with CE-based genotyping, where chances of scoring undesired alleles are minimized as has been discussed by Pan et al. (2003*a*). This is well corroborated by their high PIC values (average 0.72), whereas other detection systems have produced lower average PIC values even when genetic assessment was performed with a large number of clones from a wide range of geographical areas and suited to different agro-climatic conditions (Cordeiro et al. 2000; Singh et al. 2008). SSR markers with higher PIC values will have relatively higher probability in detecting genetic variability. However, it has been argued that the PIC value for any SSR marker is not constant and merely serves as a reference for the relative ability of that marker to detect genetic variability (Pan 2006; Singh et al. 2008; Liu et al. 2011). Recently, SSR markers developed based on EST sequences of Indian cultivars, namely CoS767 and Co1148, showed a range of PIC values from 0.12 to 0.99 (Singh et al. 2013) and an average PIC value of 0.28 with SNP markers when employed with 47 genotypes constituting many clones developed in India (Devarumath et al. 2013).

The UPGMA clustering dendrogram distinctively separated Indian cultivars from the US cultivars. Barring few outliers in some subclusters, cultivars were grouped together with high level of similarities. Two Indian cultivars, namely CoLk8001 and CoLk94184, formed a distinct cluster and showed 71% homology with a high bootstrapping value. This was expected because CoLk94184 was reported to be a self-progeny of CoLk8001 (Table 1). Along with these two cultivars, CoJ64 and Co1148 (subcluster IIa) are early maturing, high sugar bearing Indian cultivars and were separated from the major subcluster IIb wherein most cultivars exhibited >95% homology among themselves. Grouping and placement of these four cultivars in cluster I and subcluster IIa was very much similar to the cluster pattern based on 367 gene-tagged CISP marker data (96%) (Chandra et al. 2013). Though both CoJ64 and Co1148 are part of subcluster IIa, these two cultivars formed distinct nodes at 71% and 73% similarity level with the rest of the cultivars in the cluster. If closeness among cultivars is assessed based on the pedigree (Table 1), cultivars would show invariably strong relationships with their parents, as was reported earlier (Pan et al. 2007). CoLk94184 was selected from self-progeny of CoLk8001, and these two cultivars shared 69 (31%) alleles along with 6 unique alleles shared exclusively by these two cultivars. This unique pattern of allele sharing was observed with these two cultivars only. Interestingly, four common alleles from the remaining 10 Indian cultivars were not found in these two cultivars. Cultivars CoS95255, CoLk8102, and CoS97264 had one common parent (Co1158) and clustered together having average similarity of 77%. Similarly, being BO91 as one of the parents of CoS92423 and CoLk9617, these cultivars grouped together with 79% and 99% homology, respectively. However, this is not the case as observed with Co1148 and CoLk8001, as these two form distinct nodes though Co1148 being one of the parents of CoLk8001. The same phenomenon was observed on CoJ64 that shared four alleles with CoLk94184 (found only in these two), but the two cultivars clustered separately with a homology of 64%. The exceptionally well performing cultivar CoJ64 is one of the highest sugar-accumulating cultivars in Indian sugarcane history having both parents belonging to the Co series. It showed similarity to the level of 71% with the majority cultivars of cluster II possessing at least one parent of Co-series barring BO91 only. Two Indian cultivars, namely CoLk8001 and CoLk94184, together separated from the rest of the Indian and the US cultivars at similar homology level (58%) and showed the influence of the genetic background of Co-series (Coimbatore, India) from which both Indian and US commercial sugarcane cultivars were derived. Many US cultivars possessed genetic background of Co-series cultivars as revealed by a pedigree chart of US cultivars (Tew 1987, 2003). Importantly, CoLk8001 is derived from two Co-series cultivars and CoLk94184 is a self-progeny of CoLk8001, which clearly demonstrated the dominance of Co-series-based cultivars and thus showing similar level of homology and clustering with the rest of the cultivars in clusters II and III.

Clustering of the US cultivars in cluster III was mixed and was further subclustered to IIIa, IIIb, and IIIc (Fig. 2). Among these three subclusters, IIIa was the most distinct as Ho05-961 joins this subcluster as a separate node (outlier). The Ho95-998 and L99-233 cultivars showed the highest level of similarity in this subcluster. Four cultivars in subcluster IIIb formed two groups. HoCP85-845, being the parent of HoCP04-838, grouped together with HoCP04-838 at homology level of 78%. The subcluster IIIc embodied four US cultivars, and of these, LCP85-384 is the parent of the other three cultivars. Also, two cultivars, namely LCP85-384 and HoCP96-540, of this subcluster showed the maximum homology value between any two US cultivars. Contrary to this, though many Indian cultivars were more divergent among themselves, the highest similarities among any two Indian cultivars were higher (99%). One plausible reason could be that all Indian cultivars possess a high level of Co-series parentages. High levels of similarity were also observed among these lines in an earlier study using CISP markers (Chandra et al. 2013). Misnaming of identical cultivars as different cultivars is not possible, as these lines are different in appearance. Possibly, this was also the reason that the US and Indian cultivars separated into two distinct clusters (II and III) with the two cultivars of cluster I depicting bridge cultivars among these two. It clearly demonstrates not only the utility of such CE-based detection tool in identifying diversity estimates or clustering patterns, but also its importance in breeding using such distinct cultivars for better cultivars with improved agronomical traits.

Two cultivars (CoLk9606 and CoS95255) shared exactly the same SSR fingerprints, indicating a possible mislabeling of either cultivar. Since these two cultivars have different parents (Table 1), they would produce different SSR fingerprints had mislabeling not occurred. In earlier genotyping studies, identical and repeatable electrophoregarms were obtained from multiple samplings of same cultivars across locations and years (Pan et al. 2007). Moreover, sugarcane, being an asexually propagated crop, exchange plant materials through plant cutting, either whole stalks or three-bud setts. Therefore, the genetic identities of clones are maintained whenever cuttings from the same clone are planted to different locations or environments. Hence, a higher likelihood of misplacement or mislabeling of clones could result in identical capillary electrophoregrams or genetic identities among cultivars. With this technology, Pan et al. (2003*b*) have successfully identified clones that had been misidentified for some time, such as CP96-1602 or LCP85-384.

Another significant finding in the current study is the cultivar-specific SSR alleles found noticeably in Indian cultivars CoJ64, CoLK8001, CoLk94184, and CoSe92423, which demonstrate that these leading Indian cultivars are distinct from the others used in the present investigation. Of the six such alleles, three alleles, namely SMC486CG_229, SMC36BUQ_125, and mSSCIR74_216, were CoLk8001 cultivar-specific, whereas one of each was observed with cultivars CoJ64 (mSSCIR3_182), CoLk94184 (SMC334BS_154), and CoSe92423 (mSSCIR43_238) (Fig. 3; supplementary data, Fig. S1). Six alleles, which were predominantly associated with the US cultivars as reported by Pan et al. (2007), were also observed in one Indian cultivar. Of these alleles, two were observed in Co1148 (SMC334BS_161, and mSSCIR3_187), three in CoLk8001 (SMC486CG_227, mSSCIR66_130, and mSSCIR66_134), and one in CoJ64 (SMC851MS_136). Two alleles, namely mSSCIR3_202 and mSSCIR3_204, were observed in one US (L99–233) and one Indian cultivar (Co1148). Dendrogram analysis also indicated that CoLk94184 and CoLk8001

clustered together with 71% similarity, whereas CoJ64 and Co1148 formed distinct and separate subcluster IIa with 68% similarity (Fig. 2).

In total, six cultivar-specific alleles were found in CoLk8001, two in CoJ64, two in Co1148, one in CoLk94184, and one in CoSe92423. These cultivars are well known for their peculiarities, and apart from early maturing (barring CoSe92423) and high sugar accumulating ability, CoLk8001 is known for better ratooning, and Co1148 has been a dominant cultivar for almost four decades since its release in the late 1960s (Nair 2011). Co1148 is still a preferred cultivar in Madhya Pradesh, part of subtropical India. CoJ64 has been continuously used as a check in India for varietal development programs as a high sugar accumulating variety. CoLk94184 is a new variety known for its tolerance to both moisture and water lodging with up-facing deep green leaves.

Regardless of the occurrence of such cultivar-specific alleles in some cultivars identified with this particular CE-based genotyping system, their significance in breeding can be visualized only when these are deployed in the long process of evaluation of sugarcane clones. Hence, future study would be targeted to understand the sequence feature and its functional significance associated with these unique cultivars. Being an open-pollinated crop, self pollination rate of sugarcane is high unless pollen control is strictly enforced. In this regard, these cultivar-specific SSR alleles can serve as a reliable parameter to distinguish hybrids from self-progenies because visual selection of promising hybrids among cross progenies is often unreliable (Divinagracia 1980; Heinz and Tew 1987). To some extent, species- and trait-specific DNA markers have been used in sugarcane breeding (Pan et al. 2001; Pan 2006; Selvi et al. 2006; Maccheroni et al. 2009; Oliveira et al. 2009).

The significance of CE-based molecular identification of sugarcane clones has been demonstrated in US breeding programs and in making correct clonal identification (Pan et al. 2003*b*, 2007; Pan 2010). Molecular identities represented by the presence or absence of 144 SSR alleles also have become part of the variety release notes along with agronomical traits (Tew et al. 2005*a*, 2005*b*, 2011; White et al. 2011; Hale et al. 2012). The very first interspecific cross hybrid Co205 was made in 1912 from a cross between *S. officinarum* and *S. spontaneum* (Nair 2011). SSR markers are valuable in dealing with the complexity created by the interspecific hybrids. Depicting higher genetic divergence as demonstrated with a set of Indian cultivars over the US cultivars further assuages the utility of SSR capillary electrophoregrams in studying the allelic divergence, cultivar-specific alleles, and verification of breeding clones and cultivars needed during the multi-year process of sugarcane breeding. This, as well as our previous reports (Pan et al. 2007; Pan 2010), has demonstrated the scope to widen the applicability of CE-based SSR allele detection system with diverse germplasm from other parts of the world to facilitate the identification of DNA markers associated with different agronomical traits, so as to minimize the time scale involving in sugarcane breeding worldwide.

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